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# Aberrant expression of mucin core proteins and O-linked glycans associated with progression of pancreatic cancer

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## Abstract

**Purpose—**Mucin expression is a common feature of most adenocarcinomas and features prominently in current attempts to improve diagnosis and therapy of pancreatic cancer and other adenocarcinomas. We investigated the expression of a number of mucin core proteins and associated O-linked glycans expressed in pancreatic adenocarcinoma (PA) – sialyl Tn (STn), Tn, T antigen, sialyl Lewis A (CA19-9), sialyl Lewis C (SLeC), Lewis X (LeX) and sialyl Lewis X (SLeX) – during the progression of pancreatic cancer from early stages to metastatic disease.

**Experimental Design—**Immunohistochemical analyses of mucin and associated glycan expression on primary tumor and liver metastatic tumor samples were performed with matched sets of tissues from 40 autopsy patients diagnosed with PA, 14 surgically resected tissue samples, and 8 normal pancreata.

**Results—**There were significant changes in mucin expression patterns throughout disease progression. MUC1 and MUC4 were differentially glycosylated as the disease progressed from early PanINs to metastatic disease. De novo expression of several mucins correlated with increased metastasis indicating a potentially more invasive phenotype, and we demonstrate the expression of MUC6 in acinar cells undergoing acinar to ductal metaplasia. A “cancer field-effect”

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**Author Contributions:** NR conducted experiments, JA created the TMAs, EL, DD, and AL provided pathological review of samples and conceptual advice regarding study design, HW, UM and HC contributed key reagents and concepts to the work, FY performed statistical analysis, NR and MH conceived of the study and wrote the paper. All authors edited and approved of the submitted and published versions.

that included changes in mucin protein expression and glycosylation in the adjacent normal pancreas was also seen.

**Conclusions—**There are significant alterations in mucin expression and post-translational processing during progression of pancreatic cancer from early lesions to metastasis. The results are presented in the context of how mucins influence the biology of tumor cells and their microenvironment during progression of pancreatic cancer.

### Keywords

pancreas cancer; mucins; O-glycosylation; tumor microenvironment

## INTRODUCTION

Pancreatic adenocarcinoma (PA), the 4$^{th}$ leading cause of cancer-related deaths in 2010, is highly lethal because of its propensity to metastasize early in disease progression. Metastasis results from two key factors in cellular behavior: the capacity to migrate to a different location and the ability to survive and proliferate at this new location. This process requires a reconfiguration of many molecular features of the cell surface leading to changes in structural, signaling and metabolic features of the cell. Mucins are a prominent class of cell surface glycoproteins expressed by epithelial cells and cancers derived from them, which serve to configure local molecular and structural aspects of the cell surface and engage in signal transduction that informs the cell of its exterior condition and environment (1, 2). The cell surface of secretory epithelial cells and associated cancers includes proteins that have specific patterns of glycosylation and other post-translational modifications. Normal epithelial cells derived from different organ sites (such as the pancreas) express a subset of the more than 20 mucin core proteins, which are heavily O-glycosylated in a manner specific to the requirements of the epithelial cell surfaces in that organ. The process of transformation to a malignant state results in expression of different mucin core proteins with distinct patterns of complex *O*-linked glycosylation, principally to the tandem repeat domain, which in cancer includes short, truncated structures not seen in normal epithelia. Of these shortened structures, the most notable are the pan-carcinoma structures sialyl Tn (STn, NeuAcα2-6GalNAc) and Tn (GalNAc) along with a simple Core 1 glycan extension, the T antigen (T, Galβ1-3GalNAc). These shortened glycans on the mucin tandem-repeat domains create tumor-specific antigens (structures) in three ways: 1) Tn and STn are not present on normal epithelia making them unique to the tumor tissue; 2) they expose protein regions of the tandem repeat domain that are otherwise blocked to recognition by antibodies; and 3) they produce new glycopeptide structures that are rarely seen in normal adult tissues if at all.

In this report, we examined the expression of mucin core proteins and associated glycans including MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC16, MUC17, CA19-9, sialyl Lewis C (SLeC), Lewis X (LeX), sialyl Lewis X (SLeX), T, Tn, STn and three glycopeptides – Tn/STn on MUC1, Tn on MUC4, and T on MUC1 on matched sets of primary tumor and liver metastasis tissue samples from 40 autopsy patients presenting with PA, 14 resection tissue samples from those autopsy patients who received surgical treatment, and 8 normal controls. The results of this study highlight the importance of the changes on tumor epithelia as compared to its normal epithelial counterpart and provide further insight into how tumors establish a favorable microenvironment to promote survival and disease progression.

# MATERIALS AND METHODS

## Materials

All immunohistochemistry analyses were performed using Dako EnVision kits and the antibodies listed in Table 1. Mucin, glycan-specific and glycopeptide specific antibodies were purified at the monoclonal antibody facility at the University of Nebraska Medical Center. Antibodies for Hes1 and cytokeratin 19 were obtained from Abcam, PE and FITC secondary antibodies were purchased from Invitrogen, 800CW secondary antibody was purchased from LiCor, and anti-fade mounting media with DAPI was purchased from Vector Labs.

## Rapid Autopsy Patient (RAP) Samples

Pancreatic tumors, metastases, and other tissue specimens were obtained with consent and IRB approval from surgically resected samples or from decedents through the Rapid Autopsy Program at the University of Nebraska Medical Center. To ensure minimal degradation of tissue, organs were harvested within three hours post mortem and the specimens flash frozen in liquid nitrogen or placed in formalin for immediate fixation.

## Tissue Microarrays

Tissue microarrays (TMAs) were made from paraffin blocks of formalin fixed tissue from rapid autopsies, control specimens of uninvolved kidney and colon tissue, and pancreas from non-cancerous donors using 2.0 and/or 2.5 mm cores that were cut into 4 micron sections and mounted on charged slides. Tissue microarrays contained 2-4 sections from each patient's tumor and separate arrays were made for primary tumor and matching metastatic deposits in the liver. The analyses presented here employed matched sets of uninvolved tissue, primary tumor and liver metastases. Expression in tumor tissue was evaluated and scored for cancer cells and the stromal compartment by an independent pathologist (infiltrating immune cells and endothelial tissue within the tumor were considered part of the stroma).

## Tissue staining

Serial sections of tissue microarrays were stained using the primary antibodies listed in Table 1 with standard IHC procedures. Briefly, tissue arrays were deparaffinized with xylene and re-hydrated using an alcohol gradient followed by submersion in water. As needed, antigen retrieval was performed using an alkaline citrate buffer and microwave treatment. Endogenous peroxidase activity was quenched and slides were blocked with 5% BSA. Following incubation with primary and secondary antibodies the substrate-chromagen 3,3′-diaminobenzidine was added followed by counterstaining with Harris hematoxylin and dehydration with an alcohol gradient ending with xylene. Primary antibody concentrations and incubation conditions were optimized using positive control tissues.

## Tissue analysis

Histological sections were annotated by two independent pathologists. Sections were scored for differentiation as well-differentiated, well-moderately differentiated, moderately-poorly differentiated and poorly differentiated. Relative antigen expression levels were semi-quantified based on the percentage of cells of the same cell type staining positive for each antigen. A scale of 0-3 was used to indicate the relative percentage of cells positive with 0 being no detectable expression and 3 indicating that ≥67% of the total cell population expressed the antigen. Discrepancies between two pathologists were rare, and were resolved by averaging the results. 2-4 sections from each tumor were present on tissue microarrays and the final score for each sample was based on the mean score from all sections stained, to

best represent the spectrum of tumor heterogeneity. Differences in relative expression levels between the primary tumor site and corresponding liver metastasis were semi-quantified by subtracting the score for the liver metastasis from that of the primary tumor (primary tumor score – liver metastasis score). Scores and differences in scores were converted into heat maps for better visualization. Pictures were taken using a Nikon Eclipse 90i microscope at 200x magnification.

### Statistical Analysis

Comparisons between immunohistochemical scores for uninvolved samples versus tumor and primary tumor versus liver metastasis in the autopsy samples were analyzed using the signed rank test to evaluate whether the median difference equaled zero, since these were matched samples. For comparisons involving the normal tissue samples where matched-patients were not available, the Wilcoxon rank sum test was used to compare the median immunohistochemical scores between the uninvolved samples and the normal samples or between the primary tumor samples and normal samples. For all tests, the Benjamini Hochberg (BH) method was used to control false discovery rate. The antigens with BH adjusted p values less than 0.05 are considered to have significant differences in expression between groups (3).

## RESULTS

### Mucin Expression Patterns in Normal Pancreas and Primary Tumors

Cancer cells exhibit an altered glycosylation profile (4) giving rise to new tumor-specific antigens. The highly glycosylated tandem-repeat domain of mucin core proteins are particularly rich sources of tumor-associated glycan antigens, in part because of the multivalent nature of the tandem repeat and the resulting diversity of structures that are present on these molecules. It is hypothesized that these altered structures contribute to cancer progression. Supplementary Figure 1A shows a simplified, schematic diagram of the glycan structures analyzed in our studies and a general schematic of the protein structure of a trans-membrane mucin such as MUC4. The glycopeptide antigens analyzed in these studies are derived from sequences of the tandem repeat of the indicated mucin core protein glycosylated with either Tn or T. The antibodies 5E5 and 1B9 recognize the glycopeptide structures Tn/STn on MUC1 and T on MUC1 respectively. The antibody 3B11 binds to a specific Tn on MUC4 structure within the tandem repeat.

We evaluated expression of mucin core proteins and associated glycopeptides structures on matched sets of primary pancreatic tumors and liver metastases obtained from PC patients who underwent rapid autopsies (Table 2) and compared these to expression in normal pancreases (excess tissue from organ donors) (Figure 1A, Table 3). The heat map in Figure 1A provides visual representation of the immunohistochemical score for relative expression levels of each antigen on tumor cells analyzed in each autopsy patient's primary tumor, matched liver metastases, and in 8 different normal pancreases. Table 3 presents a summary of staining for the tissue sections that includes an average score for antigen expression in different normal and malignant cell types within the sections, and the number of patients that were positive for the indicated staining. There were significant alterations in the expression patterns of mucins in primary tumors as compared to normal pancreas from organ donors. Consistent with previous reports, ductal epithelial cells of normal pancreas expressed primarily MUC1 and MUC6, CA19-9 and SLeC (5-10). We detected high and consistent levels of T antigen on MUC1 in normal pancreas ductal cells, which was not detected in primary tumor cells commensurate with the appearance of STn on MUC1 by almost all primary tumors. One case of primary tumor showed T antigen expression distal to the tumor with a gradient loss of expression as one approached the tumor (data not shown). This is the

first report of nonsialylated T antigen expression in normal tissues, to our knowledge, and as such may be unique to the pancreas. We also detected the appearance of MUC4 and/or Tn on MUC4 by all primary tumors, confirming previous reports that increased MUC4 expression is correlated with the progression of pancreatic cancer (11, 12). Similarly, MUC5AC and MUC16 were expressed in a significant percentage of primary tumors, but were not seen in normal pancreas. We observed expression of LeX in the primary tumor but did not detect SLeX, which has been previously reported to contribute to tumor cell invasion and metastasis (12-14). The LeX antigen was also repeatedly observed on infiltrating immune cells, but other antigens evaluated here were rarely seen on immune and inflammatory cells. The expression of SLeC was similar to CA19-9, which was not surprising given the similarity in these two structures (SLeC differs from CA19-9 by a single fucosyl residue) (6, 15). There were no consistent antigenic signatures associated with tumor differentiation status, although there was a trend that MUC6, MUC7, LeX and T antigen were higher in well-differentiated tumors as compared to moderately to poorly differentiated tumors.

It is well documented that Tn and STn structures are among the most cancer-specific biomarkers (4-6, 10). The appearance of Tn and STn structures in cancers are due in part to the presence of mutations in (or epigenetic inactivation of) the Cosmc protein (13, 14), the core 1 synthase, or other enzymes involved in O-glycan extension. Cosmc is a chaperone that is necessary for core 1 activity and consequently the extension of Tn glycan into core 1 or core 2 structures including the T antigen. We observed an abundance of Tn and STn in pancreatic cancer tissues; however, there was also expression of extended structures of the Lewis series that are likely O-linked (Figure 1 and Supplementary Figure 1B), suggesting that Cosmc inactivation does not entirely explain the presence of Tn and STn in these samples. Additionally, the Tn antigen along with the Tn/STn on MUC1 and Tn on MUC4 were largely seen to be perinuclear within the tumor cells, though occasional surface and luminal staining of the ducts could be seen, especially with the 5E5 and 3B11 antibodies in liver metastases (Supplementary Figure 1B).

## Comparison of Expression Patterns between Primary Tumor and Liver Metastasis

Figure 1 and Table 2 show staining results in matched sets of liver metastases from autopsy patients. The progression of pancreatic cancer to liver metastasis was accompanied by alterations in mucin glycoprotein expression as shown in Figure 1A and highlighted in the comparison heat map presented in Figure 1B. Liver metastases expressed many of the same mucins and glycans as corresponding primary tumors, including as MUC4, MUC5AC and STn. However, there were significant and consistent alterations in expression of mucin core proteins within individual patients when primary tumors were compared to corresponding liver metastases (Figure 1B). In almost all cases, MUC2 and MUC5B were absent in the primary tumors but were expressed in liver metastases. Additionally, MUC4, MUC5AC, MUC16, STn, SLeC, T on MUC1 and Tn on MUC4 were more highly expressed in the liver metastases from virtually all patients. Conversely, MUC6, MUC17, and MUC7 were more highly expressed by primary tumors. The expression pattern of LeX in both the primary tumor and liver metastases was unlike any other antigen analyzed. In the primary tumor, it was predominantly expressed in the cancer cells forming duct-like structures, though it could be seen on the infiltrating immune cells in some cases. In the liver metastases, this pattern was reversed where it was predominantly found on the infiltrating immune cells and lacking in the tumor cells. We observed staining for Tn on MUC4 by most primary tumors, which was increased in liver metastases. There were also alterations in expression of STn/Tn on MUC1 and T on MUC1 between primary tumors and liver metastases. Primary tumors expressed abundant STn/Tn on MUC1 that was accompanied by low expression levels of T on MUC1; however, this pattern was reversed in liver metastases, which expressed higher

amounts of T on MUC1 (Figure 1). Notably, a few patients showed an overall downregulation of mucin expression (e.g WD34) or upregulation of mucin expression (e.g. MD4) in metastatic lesions, whereas the majority showed both upregulation and downregulation of different core proteins and associated glycan structures (Figure 1B).

## Antigen Expression in Pancreatic Resection Samples

Of the 40 autopsy patients analyzed, 14 previously underwent surgical resection upon initial diagnosis, enabling us to study mucin expression during the progression from early stage malignancy to metastatic disease. About half of the resected patients presented with high grade pancreatic intraepithelial neoplasias (PanINs) in which mucin expression patterns were annotated separately from the malignant compartments. Figure 2 displays the expression patterns from these resected samples and compares these to matched recurrent primary tumors and metastatic lesions obtained at autopsy. MUC1 and MUC4 were expressed in all of observed PanINs and MUC5AC, MUC6, Tn, SLeC and CA19-9 were present in at least 67% or more of the PanINs. Although Tn structures were detected in a number of the PanINs, they were predominantly expressed in intracellular compartments (consistent with detection of precursor structures in the ER or Golgi and not cell surface expression). MUC7 and MUC16 were absent in PanINs but were expressed in primary tumors and metastatic lesions (Figure 2) suggesting that expression of these mucins were later events in disease progression. Conversely, MUC17 was expressed by half of the PanIN lesions and corresponding malignant compartments of the resection samples; however, its expression was almost completely absent in metastatic lesions. In the resection samples, MUC6 was expressed by many of the PanIN lesions, but was absent in all malignant tumor cells (even though we sampled multiple sections from the resections). MUC6 showed heterogeneous expression in metastases evaluated at autopsy. Thus, the lack of MUC6 expression in resected samples may be due to heterogeneity of expression in pancreatic cancer, or there may be limited re-expression of MUC6 in some cases during disease progression.

## Cancer Field-effect

We evaluated the uninvolved “normal” pancreas adjacent to tumor tissue for expression of mucins and compared this to the normal pancreas and tumor samples (Figure 3 and Table 3). Expression in the ductal cells, acinar cells and islet cells were annotated separately. A number of antigens associated with tumor progression were detected in the adjoining normal tissue, including MUC4, MUC17, LeX, Tn, Tn/STn on MUC1 and Tn on MUC4. There were increases in expression of MUC1, MUC6, CA19-9, SLeC, and loss of expression of T and T on MUC1, which are present in the normal pancreas. There were also notable changes in the cellular context of mucin expression. As indicated in Table 3, levels of MUC6 were elevated in the uninvolved pancreatic ducts as compared to normal ducts. Interestingly, T antigen expression increased in the ducts but was lost in acinar cells of uninvolved pancreas. In contrast, T on MUC1 decreased in the uninvolved pancreas as compared to the normal pancreas. The overall relative expression of MUC1, CA19-9 and SLeC in the ducts did not change in the uninvolved pancreas; however, the types of cells that produced these antigens were different. In the normal pancreas, MUC1, CA19-9 and SLeC were largely restricted to the ductal cells, whereas in fields adjacent to tumor the acinar cells also produced these antigens. The uninvolved tissue in resection samples displayed a field-effect similar to that seen in the autopsy samples, albeit to a much lower degree (Figure 2B and Supplementary Figure 2). Table 4 provides a statistical comparison of antigen expression in the uninvolved tissue to the tumor tissue within the resection tissues, with statistical significance highlighted in bold.

As shown in Supplementary Figure 2, the staining patterns of the tumor antigens in the uninvolved tissue were similar in the resection and autopsy samples. This supports our conclusion that the staining observed in the uninvolved tissue in the autopsy samples was not due to non-specific binding of antibodies to degraded tissue but rather these antigens are indeed expressed in the uninvolved tissue. Nonetheless, the resection samples showed an important difference from normal pancreas: there were distinct sets of acinar cells within the uninvolved pancreas that expressed SLeX and MUC6. We evaluated these samples to determine if these cells were undergoing acinar to ductal metaplasia (ADM), which has been associated with pancreatitis and early events in transformation, by using immunofluorescence to evaluate expression of Hes1 and cytokeratin 19 (CK19), markers of ADM (15). In the case of MUC6, every acinus that expressed MUC6 also expressed CK19 (Figure 4). Hes1, however, showed an inconsistent pattern of expression in our samples compared to that seen in previous reports of ADM (data not shown). Sialyl Lewis X was not reliably detected by immunofluorescence, perhaps because differential processing of the tissue for immunofluorescence masked or eliminated the epitope. This is the first report, to our knowledge, of mucin expression in cells undergoing ADM, and raises the possibility that MUC6 may be a marker for some aspect of this process. We did not detect MUC6 or SLeX in the acini of uninvolved tissue in the autopsy samples, suggesting that this is an early event in disease progression.

## DISCUSSION

Mucin expression is a common feature of all adenocarcinomas. One function of mucins on epithelial cells is to configure their cell surface properties in a manner that protects the cells in different harsh environments and allows them to configure specific biochemical properties of the local cellular microenvironment (1, 2, 16, 17). In normal tissues, the secreted mucins form a protective layer that confers specialized molecular structures for each type of epithelia (1). This layer shields the cell surface from adverse external conditions and forms a selective biofilm that allows for the passage of specific molecules establishing a microenvironment that influences the biological properties of cells or organisms that transit this matrix (1). In addition, some cell surface associated mucins engage in signal transduction, which apprises the cell of conditions at the surface and regulates expression of genes that are related to the biological needs of the epithelia on which they are expressed (1, 2, 16, 17). Tumor cells express mucins that are associated with the epithelia from which they are derived along with new mucin core proteins and glycan structures that arise during disease progression. It is our working hypothesis that tumor cells appropriate functions associated with normal and aberrant mucin glycoproteins and use these to control the tumor microenvironment and enhance survival during the progression of pancreatic (and other) adenocarcinomas.

Here, we present an in-depth evaluation of mucins and glycans expressed in primary pancreatic cancer and matched resection and liver metastasis tissue. At least 8 different mucins were expressed in the primary tumor and/or liver metastasis along with the carcinoma associated antigens STn and Tn, the T glycans, and several Lewis blood group glycans – CA19-9(SLeA), SLeC, LeX and SLeX. The expression pattern of Lewis blood group glycans in our autopsy tumor samples suggests that the short oligosaccharides, Tn and STn, were present on highly expressed precursor mucin core proteins in the endoplasmic reticulum that contained incompletely extended structures, which resulted from overexpression of the mucin core proteins or factors other than Cosmc that influence the glycosylation of mucin core proteins. Additionally, the lack of STn in PanINs suggests that the truncation of mucin type O-glycosylation by sialylation occurs later in disease progression. However, T on MUC1 was absent in the PanIN lesions, suggesting that there was differential glycosylation of mucins during early stages of malignant transformation that

remain uncharacterized at this time. Overall, our findings demonstrate that alterations in levels and glycosylation of mucin core proteins are associated with disease progression.

The transmembrane mucins MUC1, MUC4 and MUC16 are the most widely studied mucins. MUC16, also known as CA125, is a well-established marker for ovarian cancer. Our studies confirm previous reports that MUC16 is expressed by 40-65% of pancreatic cancers (18, 19), and extend these findings by demonstrating that MUC16 expression is increased in liver metastases. MUC1 was highly expressed by nearly all primary and metastatic pancreatic adenocarcinoma lesions. Although MUC1 was expressed by the normal pancreas, the results presented here demonstrate that pancreatic tumors overexpress and produce different glycoforms of MUC1. Most normal pancreas samples produce MUC1 that contains the T antigen, whereas tumors produce MUC1 that contain Tn and STn at early stages of disease (Figures 1 & 2) and this differential glycosylation is increased in metastatic lesions to the liver. Consistent with previous reports, MUC4 is not expressed in the normal pancreas but is expressed by a high percentage of PanIN lesions and primary and metastatic pancreatic adenocarcinomas (Figures 1 & 2). Similar to MUC1, there is evidence of differential glycosylation of MUC4 in premalignant and malignant lesions in that the Tn on MUC4 epitope identified by the 3B11 antibody was highly expressed in our tissue samples whereas the 4D9 antibody, which recognizes a different Tn on MUC4 epitope, could not be detected (data not shown). The creation of these truncated glycan structures are likely the result of differential activity of specific polypeptide glycosyl transferases that create these tumor associated Tn epitopes on both MUC1 and MUC4 (20). Alternatively, mutations, silencing, or differential expression of the core 1 or core 3 glycosyltransferases (or associated subunits such as the molecular chaperone Cosmc) that extend the O glycan structure beyond the initial GalNAc residue may create these structures (Figure 1).

Differential glycosylation of MUC1, MUC4 and MUC16 are predicted to affect important tumorigenic functions associated with these molecules. Glycosylation of the extracellular domain of transmembrane mucins configure molecular aspects of the cell surface by establishing locally high concentrations of specific structures that stand alone or bind to other factors and thereby regulate cellular functions including cellular polarity, adhesion and non-adhesion, and accessibility of receptors and small molecules to the cell surface. The differential glycosylation of MUC1 in pancreatic tumors is known to induce binding to ligands different from those seen in the normal pancreas. For example, the extracellular portion of MUC1 binds to protumorigenic factors such as galectin-3 (21). Glycosylated forms of MUC1 have been shown to bind to MAG, or Siglec 4. Siglecs are a family of carbohydrate-binding proteins that recognize sialylated structures, and in adults Siglec 4 is only expressed on oligodendrocytes and Schwann cells. MUC1 binding to MAG has been shown to enhance adhesion in the context of perinerual invasion by tumor cells (22). Other Siglecs are present on distinct immune-cell populations. Several recent studies showed that binding of to mucins to immune-cells through Siglec proteins attenuate immune cell function (22-24).

Molecular interactions of cell surface receptors with MUC1 or MUC4 (which can be affected by glycosylation) influence intracellular signaling events. MUC1 associates with a number of receptor tyrosine kinases that phosphorylate the cytoplasmic tail (MUC1.CT), which in turn directly conducts signals by translocating to the nucleus in association with different regulators of transcription to affect expression of a number of genes that can influence invasion, metastasis, angiogenesis, and the microenvironment (25-27) (2, 28-34). MUC4 associates with the ErbB2 receptor to affect tumorigenic processes including proliferation, apoptosis, and EMT (2, 35-39). The differential glycosylation of MUC4 in pancreatic cancer may influence its capacity to bind the ErbB2 receptor or other receptors that affect protumorigenic signaling pathways. MUC16 exhibits similar tumorigenic

functions to MUC1 and MUC4 (40, 41); however, the shedding of its extracellular domain contributes to tumorigenic functionality (42). Thus, the relatively low amounts of MUC16 in tissue samples may be due to this shedding event, and shedding in pancreatic cancer may be facilitated by differential glycosylation of MUC16 that exposes cleavage sites.

In addition to the membrane-associated mucins, pancreatic cancers exhibit *de novo* expression of one or more of the secreted mucins MUC2, MUC5AC, MUC5B, and MUC7 (Figure 1). MUC6 is the secreted mucin expressed in normal pancreas (Figure 1). The expression of MUC5AC in PanINs and its retention throughout disease progression in most tumors suggests that this mucin may have significant roles in disease progression. Aberrant glycosylation of secreted mucins may directly affect physiological processes such as immune responses as discussed above, or it may alter the types of smaller molecules such as growth factors or trefoil factors that are bound to the mucous layer. Altered glycans have been shown to be present on a number of mucins found in circulation of cancer patients at high abundance (43, 44). We hypothesize that glycans on mucins in circulation carry with them cytokines bound through lectin type interactions. This may serve to deliver secreted factors from the primary tumor to distant organ sites to enhance systemic immune-suppression and metastasis. Yue et al recently published that circulating CA19-9 was attached to a number of different proteins including some mucins in the blood from pancreatic cancer patients (45), which is in agreement with the results presented here and unpublished data from our lab.

Our findings of altered mucin expression and glycosylation in uninvolved pancreas surrounding tumor are important observations that are not often considered in the context of tumor progression. This finding supports the concept that there are field-effects in cancer that result from tumor affecting local or distant microenvironment through factors secreted by the tumor cells, or by the reaction of the surrounding normal tissues to alterations in organ structure and function that result from tumor growth. Similar results were reported with two Tn on MUC4 epitopes in inflammatory bowel disease (IBD) and colorectal cancer (46). These results are consistent with the known relationship between inflammation and mucin expression and glycosylation. Models of inflamed airways and cancer show a strong correlation between sustained inflammation and mucin over-expression. The capacity of inflammatory mediators such as IL-1β, IL-13, TNF-α, and TGF-β to induce mucin secretion as well as alter the glycosylation patterns of these mucins is well documented (43, 47-51). Thus, signaling through the STAT3 and STAT6 pathways may be important events leading to the altered mucin production seen in pancreatic cancer.

One important translational implication of these studies is that the antigenic signatures of glycosylation patterns on mucins serve as biomarkers for diagnostic uses in pancreatic cancer. Analysis of resection samples show that altered glycoforms of MUC1, MUC4 and MUC5AC are expressed early in disease progression. This is consistent with recently published studies showing that circulating forms of MUC4 and differentially glycosylated forms of MUC5AC have promise as serum biomarkers of pancreatic cancer (11, 12, 43, 44, 52-54). Other mucins including MUC7 and MUC16 are expressed when the disease has acquired an invasive or metastatic phenotype. The use of reagents that detect specific glycopeptide structures on these mucins may aid in identifying pre-malignant or malignant lesions in biopsy specimens and should be further investigated. The possibility that auto-antibodies against glycopeptide structures on mucins develop in patients with cancer and that these can be used for early detection of cancer has been discussed previously (43, 46, 55). Our results support the hypothesis that the detection of the glycopeptides or autoantibodies to glycopeptides (e.g. STn, Tn on MUC1 or MUC4) may improve specificity for detecting cancer, and serve as markers of staging. Finally, our results support the contention that the addition of a diagnostic test for SLeC to the CA19-9 diagnostic assay

could enhance the sensitivity of this test by detecting those patients that are unable to synthesize CA19-9 structure because of a congenital lack of the fucosyl transferase that creates the SLeA structure.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Statement of Translational Relevance

Mucin expression, a common feature of most adenocarcinomas, features prominently in current attempts to improve diagnosis and therapy of pancreatic and other adenocarcinomas. We present several new discoveries regarding types of mucins and associated glycan structures that are expressed in pancreatic cancer, which are presented in the context of how mucins affect the biology of tumor cells and their microenvironment during the progression of pancreatic cancer to metastasis. We evaluated matched sets of surgically resected samples and/or matched primary and metastatic tissues obtained at autopsy from patients with metastatic pancreatic cancer. Significant and novel findings include: mucin expression in surgically resected tumor that display acinar to ductal cell metaplasia; field effects of cancer on adjoining pancreas in which there are novel changes in mucin expression by adjoining uninvolved tissue; the characterization of unique glycopeptide structures and de novo expression of secreted mucins during disease progression from early lesions to metastasis.

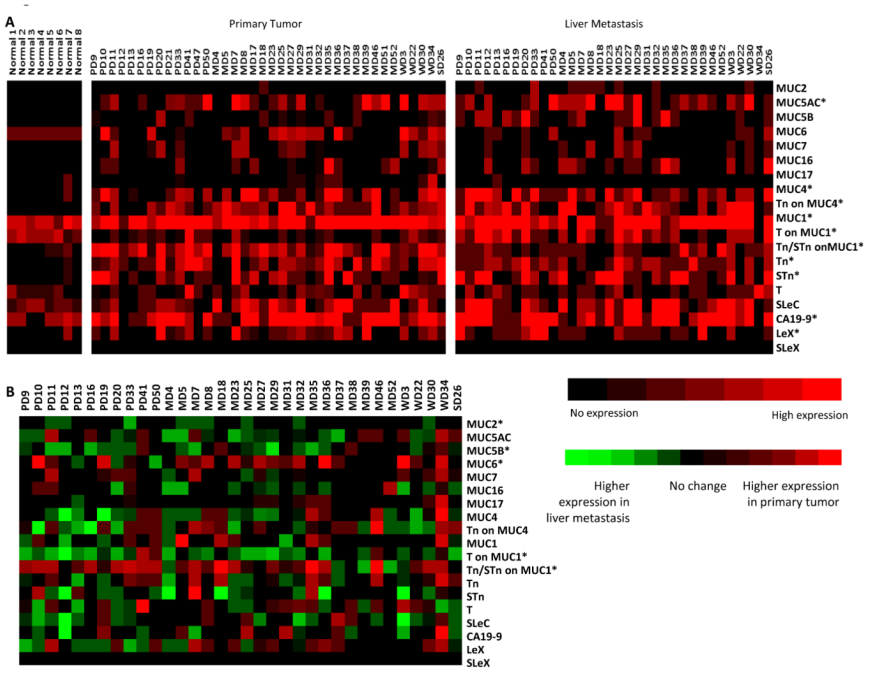


**Figure 1.**

Antigen expression profiles. (A) Heat maps show the relative expression levels of each antigen analyzed in samples from normal pancreas tissue (8 normal organ donors), and matched sets of 38 primary tumors and 34 liver metastases (4 patients did not show tumor cells in liver samples) from rapid autopsies of individual patients. The matched results for autopsy patients are presented by increasing degrees of morphological differentiation of the primary tumor (since well differentiated tumors are hypothesized to express higher quantities of mucin). Higher intensity of color corresponds to higher levels of expression based on immunohistochemical score (materials and methods). Asterisks denote statistically different antigen expression levels between the normal pancreas and the primary tumor at $p<0.05$. (B) Heat map showing comparative changes in expression levels between matched sets of primary tumor and liver metastasis. Asterisks indicate statistically significant differences in antigen expression between matched primary tumor and liver metastasis at $p<0.05$.

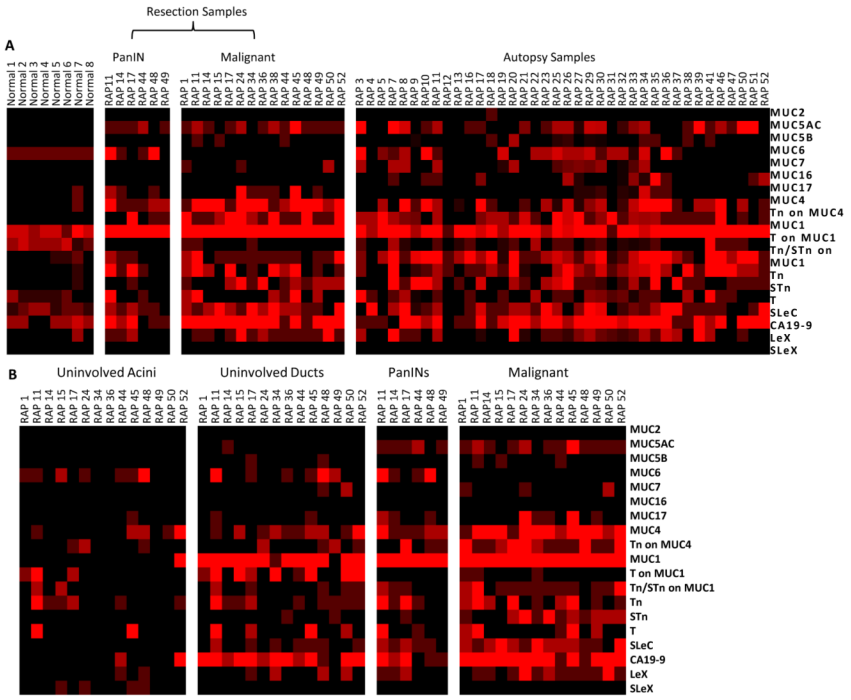


**Figure 2.**

Antigen expression patterns in areas of normal pancreas from 8 organ donors, PanIN lesions and malignant tumor from resection tissue samples, and corresponding recurring primary tumor tissues samples from autopsy patients (A). Uninvolved acini and ducts in the resection tissues were individually characterized and compared to normal tissue (B).

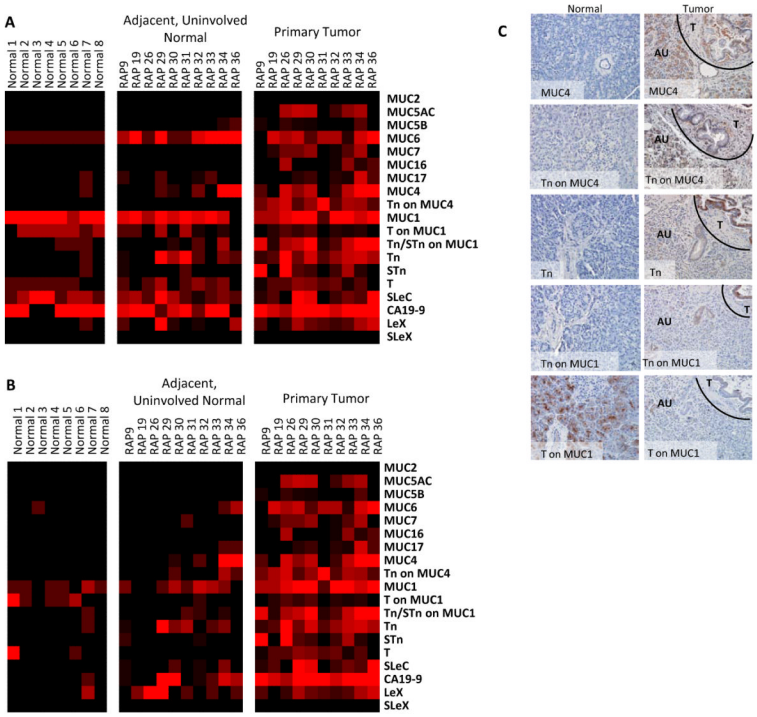


**Figure 3.**

Cancer field-effect on antigen expression. Heat maps represent changes in relative expression levels of the indicated antigens in ducts (A) and acinar cells (B) of normal and uninvolved normal tissue as compared to areas of primary tumor. (C) Immunohistochemical analysis of the field effects seen in side-by-side comparisons between normal pancreas tissues and a mixed tissue section that contains both tumor and adjacent, uninvolved tissue from patient 34. Images photographed at 200x magnification. Area of the tissue section containing the tumor is labeled with “T” whereas the adjacent uninvolved tissue is labeled with “AU.”

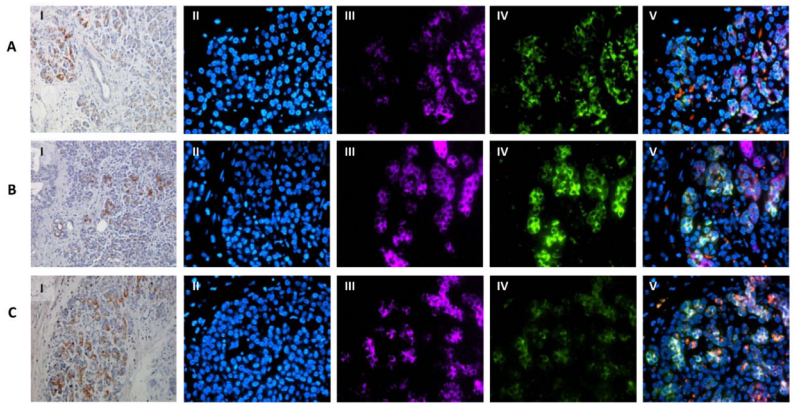


**Figure 4.**

Immunofluorescence staining of MUC6 and cytokeratin 19 in uninvolved acini from autopsy patients 15(A), 45 (B) and 44 (C), showing evidence of acinar to ductal metaplasia. Immunohistochemical staining for MUC6 on adjacent sections (I) is provided to show tissue structure, followed by DAPI (II), cytokeratin 19 (III), MUC6 (IV), and overlay (V) images. 200x magnification.

**Table 1**

List of the antibodies used for IHC analysis and the publications that characterize their epitopes for all non-commercial antibodies.

| Antigen | Antibody | Source |
|---|---|---|
| MUC1 | AR20.5 | Quest PharmaTech |
| MUC2 | PMH1 | (56) |
| MUC4 | 8G7 | (52) |
| MUC5AC | CLH2 | (57) |
| MUC5B | Panh2 | (58) |
| MUC6 | CLH5 | (59) |
| MUC7 | Panh3 | (58) |
| MUC16 | AR43.13 | Quest PharmaTech |
| MUC17 | SN 1139-2 | (60) |
| CA19-9 | NS19.9 | ATCC |
| SLeC | DuPan2 | (7) |
| LeX | P12 | Santa Cruz |
| SLeX | CSLEX | ATCC |
| Tn | 5F4 | (61) |
| STn | TKH2 | (62) |
| T | 3C9 | Henrik Clausen |
| T on MUC1 | 1B9 | (63) |
| Tn/STn on MUC1 | 5E5 | (64) |
| Tn on MUC4 | 3B11 | Hans Wandall |
| Tn on MUC4 | 4D9 | (49) |

**Table 2**

Clinical data on the autopsy patients

| RAP # | Age | Gender | Metastatic Sites (Organs) | Differentiation Grade |
|---|---|---|---|---|
| 1 | 53 | M | 0 | poorly |
| 3 | 78 | F | 5 | well |
| 4 | 59 | M | 2 | moderate |
| 5 | 65 | F | 4 | moderate |
| 7 | 71 | M | 4 | moderate |
| 8 | 72 | M | 5 | moderate |
| 9 | 69 | M | 13 | poor |
| 10 | 74 | M | 4 | poor |
| 11 | 80 | M | 6 | poor |
| 12 | 82 | M | 9 | poor |
| 13 | 72 | M | 6 | poor |
| 14 | 64 | M | 0 | moderate |
| 15 | 61 | M | 0 | moderate |
| 16 | 70 | M | 4 | poor |
| 17 | 63 | F | 7 | moderate |
| 18 | 82 | M | 8 | moderate |
| 19 | 65 | M | 6 | poor |
| 20 | 77 | M | 4 | poor |
| 21 | 60 | F | 2 | poor |
| 22 | 65 | F | 1 | well |
| 23 | 76 | F | 2 | moderate |
| 25 | 74 | M | 5 | moderate |
| 26 | 48 | M | 7 | well |
| 27 | 60 | M | 4 | moderate |
| 29 | 80 | F | 7 | moderate |
| 30 | 55 | M | 4 | well |
| 31 | 79 | M | 4 | moderate |
| 32 | 59 | M | 9 | moderate |
| 33 | 50 | M | 4 | poor |
| 34 | 81 | M | 5 | well |
| 35 | 62 | M | 3 | moderate |
| 36 | 80 | F | 4 | moderate |
| 37 | 58 | M | 9 | moderate |
| 38 | 69 | F | 12 | moderate |
| 39 | 75 | M | 10 | moderate |
| 41 | 72 | M | 8 | poor |

| RAP # | Age | Gender | Metastatic Sites (Organs) | Differentiation Grade |
|---|---|---|---|---|
| 44 | 58 | F | 2 | moderate |
| 45 | 58 | F | 7 | well |
| 46 | 78 | F | 2 | moderate |
| 47 | 72 | F | 1 | poor |
| 48 | 85 | F | 3 | moderate |
| 49 | 72 | M | 2 | poor |
| 50 | 47 | F | 15 | poor |
| 51 | 57 | M | 3 | moderate |
| 52 | 67 | M | 7 | moderate |

**Table 3**

The average immunohistochemical score for each antigen analyzed in the primary tumor, liver metastasis, uninvolved tissue, and normal pancreas. Each tissue type was further sub-divided into different cellular compartments. The number of tissues that were positive out of the total tissue samples available for each antigen in each tissue type is indicated in parenthesis under the immunohistochemical score. For example, for MUC1 there were a total 44 tumors that had a stromal compartment and of those 29 stained positive (29/44).

| | Average Immunohistological Score | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **MUC1** | **MUC2** | **MUC4** | **MUC5AC** | **MUC5B** | **MUC6** | **MUC7** | **MUC16** | **MUC17** | **CA19-9** | **SLeC** | **LeX** | **SLeX** | **Tn** | **STn** | **T** | **T on MUC1** | **Tn/STn on MUC1** | **Tn on MUC4** |
| *Primary Tumor* | | | | | | | | | | | | | | | | | | | |
| Cancer | 2.64 (45/45) | 0.04 (1/45) | 1.35 (31/45) | 1.05 (27/45) | 0.25 (10/45) | 1.35 (23/45) | 0.77 (14/44) | 0.48 (13/45) | 0.25 (8/45) | 2.32 (41/45) | 1.51 (35/45) | 1.02 (28/45) | 0.00 (0/45) | 1.43 (35/45) | 1.25 (30/45) | 0.79 (23/45) | 0.55 (20/45) | 2.05 (41/45) | 1.67 (31/44) |
| Stroma | 1.38 (29/44) | 0.00 (0/44) | 0.44 (9/41) | 0.00 (0/44) | 0.02 (1/44) | 0.00 (0/44) | 0.00 (0/44) | 0.02 (1/44) | 0.01 (1/45) | 1.33 (33/45) | 0.46 (14/45) | 0.52 (20/45) | 0.00 (0/45) | 0.07 (4/45) | 0.52 (11/45) | 0.34 (5/45) | 0.19 (5/45) | 0.02 (1/45) | 0.26 (9/45) |
| Uninvolved | 2.64 (11/14) | 0.00 (0/11) | 1.25 (6/15) | 0.00 (0/11) | 0.05 (4/14) | 1.85 (12/15) | 0.11 (1/15) | 0.00 (0/12) | 0.70 (8/15) | 1.85 (12/15) | 1.08 (11/15) | 1.44 (11/15) | 0.00 (2/12) | 0.90 (8/15) | 0.17 (4/15) | 1.00 (9/15) | 0.85 (7/15) | 0.10 (4/15) | 0.55 (4/11) |
| *Liver Metastasis* | | | | | | | | | | | | | | | | | | | |
| Cancer | 2.66 (41/41) | 0.34 (9/43) | 1.86 (35/42) | 1.29 (28/43) | 0.90 (19/43) | 0.46 (12/42) | 0.50 (11/42) | 0.93 (17/42) | 0.04 (1/42) | 2.43 (39/42) | 2.18 (37/42) | 0.93 (26/43) | 0.00 (0/42) | 1.36 (35/42) | 1.78 (30/42) | 0.80 (20/44) | 0.76 (22/44) | 1.93 (39/42) | 1.89 (30/42) |
| Stroma | 0.85 (34/41) | 0.00 (0/42) | 0.04 (2/42) | 0.04 (1/42) | 0.00 (0/42) | 0.00 (0/42) | 0.00 (0/42) | 0.00 (0/42) | 0.14 (3/42) | 1.32 (29/42) | 0.36 (7/42) | 0.64 (23/42) | 0.00 (0/42) | 0.04 (2/42) | 0.25 (5/42) | 0.04 (1/42) | 0.00 (0/42) | 0.29 (8/42) | 0.14 (2/42) |
| Uninvolved | 2.36 (17/22) | 0.00 (0/28) | 1.29 (7/28) | 0.00 (0/27) | 0.00 (0/28) | 0.00 (0/28) | 0.00 (0/27) | 0.00 (0/27) | 0.00 (0/27) | 1.79 (18/27) | 0.64 (7/27) | 0.59 (7/29) | 0.00 (0/28) | 0.00 (0/27) | 0.00 (0/28) | 0.00 (0/28) | 0.00 (0/27) | 0.07 (1/27) | 0.80 (0/28) |
| *Normal Pancreas* | | | | | | | | | | | | | | | | | | | |
| Ductal | 2.86 (8/8) | 0.00 (0/8) | 0.14 (2/8) | 0.00 (0/8) | 0.00 (0/8) | 1.00 (8/8) | 0.29 (2/8) | 0.00 (0/8) | 0.00 (0/8) | 2.14 (7/8) | 1.67 (8/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.50 (5/8) | 1.60 (7/8) | 0.29 (2/8) | 0.00 (0/8) |
| Acinar | 0.71 (5/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.14 (1/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.75 (2/8) | 1.40 (5/8) | 0.00 (0/8) | 0.00 (0/8) |
| Islet | 0.43 (3/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) |

| | Average Immunohistological Score | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **MUC1** | **MUC2** | **MUC4** | **MUC5AC** | **MUC5B** | **MUC6** | **MUC7** | **MUC16** | **MUC17** | **CA19-9** | **SLeC** | **LeX** | **SLeX** | **Tn** | **STn** | **T** | **T on MUC1** | **Tn/STn on MUC1** | **Tn on MUC4** |
| *Uninvolved Pancreas* | | | | | | | | | | | | | | | | | | | |
| Ductal | 2.70 (8/14) | 0.00 (0/11) | 0.88 (5/15) | 0.00 (0/11) | 0.15 (4/14) | 2.27 (12/15) | 0.00 (0/15) | 0.00 (0/12) | 0.30 (6/15) | 2.25 (11/15) | 1.45 (11/15) | 0.68 (6/15) | 0.00 (0/12) | 1.18 (7/15) | 0.13 (3/15) | 1.02 (9/15) | 0.76 (7/15) | 0.39 (1/15) | 0.67 (3/11) |
| Acinar | 1.24 (11/14) | 0.00 (0/11) | 0.75 (5/15) | 0.00 (0/11) | 0.00 (0/11) | 0.30 (3/15) | 0.10 (1/15) | 0.00 (0/12) | 0.20 (2/15) | 1.22 (7/15) | 0.32 (4/15) | 1.05 (8/15) | 0.18 (2/12) | 0.93 (6/15) | 0.10 (2/15) | 0.00 (0/15) | 0.07 (1/15) | 0.24 (3/15) | 0.00 (0/11) |
| Islet | 0.36 (5/14) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (8/8) | 0.00 (0/8) | 0.00 (0/8) | 0.04 (1/11) | 0.20 (3/11) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.61 (3/11) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8) | 0.00 (0/8 | 0.71 (4/7) |

## Table 4

Statistical comparison of IHC scores between the uninvolved and the tumor tissue within the resection samples. The median value listed is the median difference between the uninvolved and matched tumor samples. The p value listed is the BH adjusted p value.

| Whipple Uninvolved vs Malignant Compartment | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Antigen | median | p value | | Antigen | median | p value |
| Acinar Cells | **Tn on MUC4** | −1 | 0.0035 | Ductal Cells | **Tn on MUC4** | −1 | 0.0040 |
| | **SLeA** | −3 | 0.0040 | | SLeA | 0 | 0.6667 |
| | **LeX** | −1 | 0.0064 | | LeX | −1 | 0.0972 |
| | **MUC1** | −3 | 0.0035 | | MUC1 | 0 | 0.6667 |
| | MUC16 | 0 | 1.0000 | | MUC16 | 0 | 1.0000 |
| | MUC17 | 0 | 0.1184 | | MUC17 | 0 | 0.2679 |
| | MUC2 | 0 | 1.0000 | | MUC2 | 0 | 1.0000 |
| | **MUC4** | −2 | 0.0035 | | **MUC4** | −1 | 0.0035 |
| | **MUC5AC** | −1 | 0.0035 | | **MUC5AC** | −1 | 0.0040 |
| | MUC5B | 0 | 0.4091 | | MUC5B | 0 | 1.0000 |
| | **MUC6** | 0.5 | 0.0375 | | MUC6 | 0 | 0.0703 |
| | MUC7 | 0 | 0.4091 | | MUC7 | 0 | 1.0000 |
| | SLeX | 0 | 0.2250 | | SLeX | 0 | 1.0000 |
| | **STn** | −1.5 | 0.0040 | | **STn** | −1 | 0.0040 |
| | T | 0 | 0.4091 | | T | 0 | 1.0000 |
| | T on MUC1 | 0 | 0.5192 | | T on MUC1 | 1.5 | 0.0117 |
| | Tn | −0.5 | 0.1094 | | Tn | 0 | 0.4106 |
| | **Tn/STn on MUC1** | −1 | 0.0149 | | **Tn/STn on MUC1** | −1 | 0.0251 |